Novel Nanoformulations for Selected Mosquito Larvae Vector Control

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Mosquitoes transmit a number of diseases such as malaria, filariasis, chikungunya, dengue, Japanese encephalitis etc., causing millions of deaths every year and are the most important group of insects in terms of public health. According to WHO report of year 2009, two fifth of world population is under risk of dengue infection and in year 2010, 28,292 cases of infection and 108 deaths were reported to be caused by dengue in India (NVBDCP, 2011). Figure of malaria is much higher than dengue affecting 225 million and 7,81,000 deaths worldwide in 2009: whereas, 1.49 million infection and 767 deaths were reported in India in 2010 (NVBDCP, 2011)

In recent years, repeated use of synthetic insecticides for mosquito control has disrupted natural biological control systems and led to resurgences in mosquito populations leading to major outbreaks of mosquito borne diseases. Moreover, development of resistance and undesirable effects at various trophic levels in food chains has fostered environmental and human health concerns which initiated a need to search for alternative control measures.

Nanoparticles have attracted considerable attention, owing to their various applications particularly silver nanoparticles, which are reported to possess antibacterial, antifungal, anti-inflammatory, anticancerous and anti-viral activity. Nanotechnology is mainly concerned with synthesis of nanoparticles, nanocomposites etc. of variable size, shapes, and most predominantly studied nanoparticles today are those made from noble metals, *viz.* Ag, Pt, Au and Pd. Among these, silver nanoparticles (AgNPs) play a significant role in the field of biology and medicine. Besides chemical reduction process, biosynthesis of silver nanoparticles has also been attempted using plants, bacteria, fungi etc. which are known to reduce silver ions into silver nanoparticles by both extra and intra cellular processes. Application of plants for synthesis of nanoparticles can be advantageous over other biological processes because it get rid of the complex process of maintaining cell cultures and is also suitable for large-scale nanoparticle synthesis. Green nanoparticle synthesis has been accomplished using biocompatible plant extract, reducing and capping agents.

Nanoemulsions are part of an extensive class of multiphase colloidal dispersions having uniform and extremely small droplet sizes, typically in the range of 20–200 nm. They are kinetically stable liquid dispersion of an oil phase and a water phase, in combination with a surfactant having low viscosity and optical transparency. In the world of nanomaterials, nanoemulsions are deformable nanoscale droplets that have viscosity varying from liquid to highly solid and optical properties ranging from opaque to nearly transparent. The formation of nanoemulsions takes place when an external shearing force is applied to rupture larger droplets into smaller ones. Although nanoemulsions have destabiltity, still they can persist over many months or years due to the presence of a stabilizing surfactant that inhibits the coalescence of the droplets (Walstra, 1996). Most of the mechanical properties of nanosystems are due to the high surface to volume ratio, which can make the particles very reactive or catalytic; moreover their dispersal and reaction with fast ions transport. There is substantial evidence that catalytic activity of nanomaterials also opens potential risks in their interaction with biomaterials, thus producing reactive oxygen species (ROS) (Park, 2009) which might underlie and explain both the antibacterial activity of nanosilver and its potential toxicity to humans.

The toxic effects of AgNPs are related to the release of free silver ions. Toxic compounds adversely affect organisms when they hinder with their regular physiological processes. Nanotoxicology takes up this challenge to work out the molecular events that regulate bioaccumulation and toxicity of nanoparticles. Toxicological nanosilver issues are still unanswered commenting that assessment of AgNPs toxicity is a two-fold problem.

- First, the inherent toxicity of NPs arises from having the same dimensions as biological molecules (e.g. DNA and proteins, 2 nm) and thus they may directly interact to damage DNA, denature proteins and enzymes and produce free radicals (Hsin *et al*., 2008).

- Secondly, the toxicity of elemental silver and biologically active silver ions is due to the extensive surface area which helps them to act as channel for various metabolic pathways.

Recently, nanopreparations including silver and gold nanoparticles, nanoemulsions and nanoencapsulations have acquired considerable attention owing to their mosquito larvicidal activity. Extensive literature is available on their bioefficacy evaluation against common mosquito vectors however, no report exists on their toxic effects to reveal possible cause of mortality regarding mode of action. Hence, the study is planned to gain an insight on the toxic effects of various nanoprepartions by comparing their efficacy with emphasis on cytotoxic, genotoxic and neurotoxic effects in selected mosquito vectors.

LITERATURE REVIEW

Mosquitoes are major public health concern at global level as they act as vectors for transmitting a number of diseases in humans. Mosquito larvae are attractive targets for devising effective control measures. Conventional methods for their control involve the use of chemical insecticides which leads to the development of resistance hence results in disease outbreaks. Nanomaterials are emerging as fastest growing materials for controlling theses vectors due to their unique physical and chemical properties due to extremely small size and high surface area.

Among different nanomaterials, silver nanoparticles (AgNPs) are most commercialized due to their wide applications ranging from antimicrobial, biomedical, insecticidal, agriculture, biosensor and water purification. Silver nanoparticles have received considerable attention as antimicrobial agents and have been shown to be an effective antimicrobial agent.

Synthesis of silver nanoparticles has been reported by a number of methods involving chemical, physical and biological synthesis. Dragieva *et al*. (1999) synthesized and characterized silver nanoparticles using transmission electron microscopy (TEM) and X-ray diffractometry. TEM analysis showed that AgNPs were spherical in shape and their size ranged from 40 to 60 nm. X-ray diffractometry specified that the sample was crystalline and had a face centered cubic structure of pure silver. Junjie *et al*. (2000) prepared silver nanoparticles of different shapes (spheres, rods, and dendrites) by using a pulse sonoelectrochemical technique from an aqueous solution of $AgNO_3$ in the presence of nitrilotriacetate $N(CH_2COOH)_3$-NTA. The silver nanoparticles were characterized by using TEM, X-ray diffraction, and absorption spectroscopy. It was found that the concentration of $AgNO_3$ and NTA affects the shape of the nanoparticles. Baker *et al*. (2005) created nanometer sized silver particles by inert gas condensation and co-condensation techniques. The antibacterial competence of the nanoparticles was inspected by introducing the particles into a media containing *Escherichia coli*. The silver nanoparticles exhibit antibacterial effects at low concentrations. It was found that smaller particles with a larger surface to volume ratio provided a more efficient means for antibacterial activity.

A variety of chemical approaches have also been utilized to produce silver nanoparticles with different size distribution and different shapes (Prabhu *et al.*, 2010, Sadeghi et *al.*, 2010, Hussain

et al., 2011). Das *et al*. (2011) prepared uniform silver nanoparticles through the chemical reduction of silver ions by ethanol in presence of sodium linoleate and checked antibacterial activity. Dong *et al*. (2012) chemically synthesized nanoparticles using $NaBH_4$ and PVP as reducing and stabilizing agents, respectively. Chemically synthesized AgNPs had been synthesized using different chemical methods and tested for their antibacterial potential however, no report is available on their mosquito larvicidal potential. Therefore, in the present study their effect would be evaluated in selected mosquito vectors.

Chemical methods are usually employed for nanoparticles synthesis at industrial level which involves the use of toxic reducing and capping agents for synthesis thus limiting their use in biomedical applications due to risk of health hazard. Therefore, recently the interest is shifted towards utilizing potential of biological agents (plants, bacteria, fungi etc.) for nanoparticles production.

In the recent past, several plants have screened successfully for silver nanoparticles synthesis like *Azadirachta indica* (Shankar *et al.,* 2004), *Emblica officinalis* (Ankamwar *et al.,* 2005), *Aloe vera* (Chandran *et al.,* 2006). Albrecht *et al*. (2006) highlighted the scope of green chemistry in context to potential health effects of nanoparticles, along with medical applications of nanoparticles including imaging, drug delivery, disinfection, and tissue repair. Burd *et al*. (2007) testified that materials in nano-size range may have properties different than those bulk materials of the same composition. This allows them to perform exceptional feats of conductivity, reactivity and optical sensitivity: causing harmful interaction with biological systems and the environment, with the latent to generate toxicity. Mohanpuria *et al*. (2008) critically assessed the role of microorganisms and plants in the synthesis of nanoparticles. Xia *et al.* (2009) have reviewed the use of plants or their extracts in the synthesis of silver and gold nanoparticles for various human applications. Karla *et al.* (2010) worked on the biomedical aspects of nanosilver. Elumalai *et al*. (2010) prepared silver nanoparticles using aqueous extract of shade dried leaves of *Euphorbia hirta*. UV-visible spectroscopy studies were carried out to trace the formation of Ag nanoparticles. Scanning Electron Microscopic (SEM) was employed to characterize the Ag nanoparticles. SEM image divulges that silver nanoparticles are quite poly-dispersed and their size ranges from 40 nm to 50 nm. These silver nanoparticles were found to be effective against

Bacillus cereus and *Staphylococcus aureus*. The step towards extracellular synthesis of Ag nanoparticles using dried biomass appears to be cost effective, eco-friendly than the conventional methods of nanoparticles synthesis. Kirthi *et al*. (2011) observed assessments of the antiparasitic activities to determine the efficacies of synthesized silver nanoparticles (AgNPs) using aqueous leaf extract of *Mimosa pudica* against the larvae of malaria vector, *Anopheles*, filariasis vector *Culex quinquefasciatus* and *Rhipicephalus (Boophilus) microplus*. Santhoshkumar *et al*. (2011) produced silver nanoparticles using *Nelumbo nucifera* leaf extract and checked its larvicidal activity against malaria and filariasis vectors. Kamaraj *et al*. (2012) amalgamated silver nanoparticles (AgNPs) utilizing aqueous leaf extract from *Musa paradisiaca* (Musaceae), and its acaricidal and larvicidal efficacy was tested. Patil *et al*. (2012) monitored larvicidal activity of silver nanoparticles: synthesized using *Plumeria rubra* plant latex against *Aedes aegypti* and *Anopheles stephensi*. Borase *et al.* (2013) gave an account on phyto-synthesized silver nanoparticles as potent mosquito biolarvicidal agent. These phytosynthesized silver nanoparticles as a mosquito larvicidal agent are gaining importance instead of chemical insecticide because of their safety, less harmful effect to non-targeted species.

Besides nanoparticles, nanoemulsions are now being identified as promising delivery systems. Only a few efforts have been made recently for the preparation of nanoemulsion from essential oils and tested for their efficacy against mosquito vectors. Wang *et al.* (2007) proposed a two-step course for formation of nanoemulsions in the system water/poly (oxyethylene) nonionic surfactant/methyl decanoate at 25^{o}C. In this procedure all the components were mixed at a certain composition to prepare a microemulsion concentrate, which was rapidly subjected into a large dilution into water to generate an emulsion. Bluish transparent oil-in-water (O/W) nanoemulsions were formed. Anjali *et al*. (2011) formulated nanoemulsion composed of neem oil and non-ionic surfactant Tween 20, with a mean droplet size ranging from 31.03 to 251.43 nm. The larvicidal effect of the formulated neem oil nanoemulsion was checked against *Culex quinquefasciatus*. Nanoemulsions have not been evaluated for their possible toxic effects in any animal models and this task would be taken up in the present study.

Silver nanoparticles have gained much popularity recently owing to the broad spectrum of antimicrobial activity. Very few reports on the toxicity of silver nanoparticles are available.

Anderson *et al*. (1994), Matsuura *et al*. (1997) Gupta *et al*. (1998) and Cadet (1999) reported prolonged antimicrobial activity of nanoparticles and their toxic effect on environment and oragnisms. Sorensen *et al*. (2003) hypothesized that the toxic effects of nano-silver are due to a combination of the specific properties of silver nanoparticles and the generation of ions from them. They formulated '0-hypothesis' which states that toxic effects of nano-silver are proportional to the activity of free silver ions released by the nanoparticles. Turrens (2003) discussed the toxicological potential of nano-materials (NMs) by comparing the possible injury mechanisms and adverse health effects of engineered and ambient ultrafine particles. This consideration leads to assessing toxicological potential as well as promoting safe design and use of NMs. Boonstra (2004) described briefly the generation of reactive oxygen species (ROS) and their effects on cell cycle progression, signal transduction cascades, protein ubiquitination and degradation, and the cytoskeleton. At high concentrations of ROS damage was caused to the building blocks of the cell, leading to apoptosis or even necrosis. Braydich-Stolle *et al*. (2005) studies have shown that nanosilver is cytotoxic and genotoxic to several different cell lines, including NIH3T3 mouse fibroblasts, THP-1 monocytes, BRL 3A rat liver cells and C18-4 male mouse germline cells. Zhao *et al*. (2007) stated the assumptions and challenges in the nanotoxicity field and provide an underlying principle for *in vivo* animal studies to evaluate nanotoxicity. Hsin *et al*. (2008) suggested that toxicity of nanoparticles (NP) is because of its interaction with mitochondria and induction of the apoptosis pathway (*via* the production of ROS, which leads to cell death). Carlson *et al*. (2008) identified a relationship between NP size and inhibitory effects on mitochondria. They state that smaller NPs of 15 nm significantly are more toxic than larger NPs of 55 nm. Kim (2008) reported that inhalation and oral toxicity of NP in Sprague-Dawley rats have shown low concentrations induce toxicity only after 28 days of exposure. Thus, long-term exposure to silver NPs in aerosols or food packaging might pose toxicity problems in humans. Wijnhoven *et al*. (2009) made an inventory to identify knowledge gaps that have to be filled before risks for both man and the environment. It was monitored that the toxic effects of nano-silver are due to a combination of the specific properties of silver nanoparticles and the generation of ions from them. Our study aims to unravel the possible mechanism leading to the morality of mosquito larvae in terms of cytotoxic, genotoxic and neurotoxic effects of nanoformulations in selected mosquito vectors as animal models.

OBJECTIVES

The following objectives will be undertaken for the study:

- Fabrication and characterization of nanoformulations
- Evaluation of bioefficacy of nanoformulations against selected mosquito vectors
- Evaluation of toxic effects of nanoformulations in selected mosquito vectors
- Risk assesment of nanoformulations in selected non target organisms

MATERIAL AND METHODS

Fabrication and Characterization of nanoformulations:

A number of nanopreparations will be done in order to evaluate their efficacy and toxic effects on mosquito vectors. Firstly, silver nanoparticles would be synthesized chemically using $NaBH_4$ and PVP as reducing and stabilizing agents, respectively. An aqueous solution of trisodium citrate (0.5 ml, 30 mM) will be added into a flask (50 ml deionized water), and then, an aqueous solution of $AgNO_3$ (1 ml, 5 mM) would be added. Freshly prepared $NaBH_4$ aqueous solution (0.5 ml, 50 mM) would be quickly added, and the suspension immediately will turn into light yellow color. After 30s, an aqueous solution of PVP (0.5 ml, 5 mg/ml) will be added. The suspension will be changed to a darker yellow color after reaction had proceeded for another 30 min. These silver particles will be marked PVP-AgNPs (Dong *et al.*, 2012).

A number of plants would be screened for the green synthesis of silver nanoparticles. The collected plant parts will be washed thrice with tap water and twice with distilled water to remove the adhering contaminants. About 10 g of various plant leaves would be finely cut into small pieces and boiled with 100 ml of Milli Q water separately for 5 min in microwave. The boiled extracts would be filtered through Whatman no. 1 filter paper. A total of 10 ml of collected filtrate will be mixed with 90 ml of silver nitrate aqueous solution (1 mM) and incubated at room temperature, resulting in the formation of brownish yellow color indicating the synthesis of silver nanoparticles. UV–VIS spectra of silver nanoparticles aliquots will be monitored as a function of time of reaction on a double beam UV-VIS spectrophotometer in 300–700 nm range. This colloidal solution will be centrifuged at 12,000 rpm for 20 min, and pellets would be dispersed in sterile distilled water. The centrifugation and redispersion would be repeated three times to ensure the complete separation of nanoparticles.

Nanoemulsions will also be prepared to compare their efficacy and toxic effects against mosquitoes. Neem oil (with known azadirachtin concentration, technical grade), Tween 80 (Polyoxyethylene sorbitan monooleate) and Milli Q water will be used for the preparation of nanoemulsions. Tween 80 would be used as a non- ionic surfactant, which is less affected by pH and ionic strength. The emulsions will be sonicated for 1-2 hours using high energy sonication. Nanoemulsions will be prepared from varying ratios of neem oil and surfactant i.e. 1:1, 1:2, 1:3, 1:4 and 1:5 in order to optimize protocol for smallest droplet size. In the present study

nanoemulsions from neem oil will be prepared using high energy method i.e. ultrasonicator or homogenizers.
If the time permits effort would also be made to formulate nanoencapsulation.

The size and shape of these silver nanopreparation will be characterized using Transmission electron microscopy(TEM) whereas, surface functionality characteristics would be determined with the help of Scanning electron microscopy (SEM), Fourier transform infrared spectroscopy *(*FTIR*),* X-ray diffraction (XRD), Atomic force microscopy(AFM), Zeta potential analyzer etc.

Collection and maintenance of immature stages of mosquitoes:

The mosquito larvae (*Anopheles stephensi*, *Aedes aegypti* or *Culex quinquefasciatus*) will be collected from natural oviposition sites using the standard dipping procedure from district Agra (27º 10' N and 78º 05' E) and subjected to microscopic identification which would be confirmed by RAPD based markers in the Parasitology Laboratory of DEI, Dayalbagh, Agra. Larvae will be reared to the fourth instar and then used for efficacy study.

Larvicidal activity of Nanoformulations:

The larvicidal activity would be assessed by the standard procedure of WHO with some modification if required (WHO, 1996). The efficacy of prepared nanoformulations would be performed by placing 20 IV instar mosquito larvae into 200 mL of distilled water in a 250 mL beaker (Borosil). From the stock nanopreparations, desired test concentrations will be prepared and each test would include a set control group with five replicates for each individual concentration. A positive control with permethrin and a negative control will be run with each set. Mortality will be assessed after 24 h or 48 h or 72 h to determine the acute toxicities on fourth instar larvae of selected mosquito species.

Dose–response bioassay:

During the laboratory trial, synthesized AgNPs and nanoemulsion will be subjected to a dose–response bioassay for larvicidal activity against selected mosquito vectors. Different concentrations for synthesized AgNPs would be prepared for larvicidal activity. The numbers of dead larvae will be counted at different time intervals *viz.* 24 h, 48 h or 72 h of exposure, and the

percent mortality will be recorded from the average of five replicates. The mortality data would be subjected to probit analysis for calculating LC_{50}, LC_{90} and statistics at 95% upper and lower fiducial limits (Finney, 1971). Prior to analysis, mortality in treatments would be corrected for that in controls using Abbott formula (Abbott, 1925).

Evaluation of toxic effects on mosquito vectors:

i. Cytotoxicity study

In order to study biodistribution of silver nanoparticles in the cells and subcellular damage if any, due to exposure with nanoformulations Transmission electron microscopy would be done and possible cytotoxic effects would be measured.

Control and treated larvae of *Anopheles stephensi* would be fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 12 hours at 4°C. After wash in buffer, the samples will be postfixed in 1% Osmium tetra oxide for 2 hour at 4°C. The samples will be dehydrated in an ascending grade of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1 µm) would be cut with an ultramicrotome, mounted on to glass slides, stained with aqueous toluidine blue and observed under a light microscope for gross observation of the area and quality of the tissue fixation. For electron microscope examination, thin sections of grey-silver colour interference (70-80 nm) would be cut with a glass knife and mounted onto 300 mesh- copper grids. Sections will be stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a transmission electron microscope. Photographs will be digitally acquired by using a CCD camera attached to the microscope at different magnifications. The sections prepared for TEM may also be used for visualizing distribution of AgNPs through Scanning transmission electron microscopy (STEM).

The work may further be extended to see the effect on oxidative stress and reactive oxygen species generation using available kits.

ii. Genotoxicity study

Genotoxic effects will be determined in terms of DNA damage which would be studied through Random amplified polymorphic DNA (RAPD- PCR) analysis using previously established markers in the laboratory (Gupta and Preet, 2012). Briefly, the RAPD reaction would be performed in a 0.2 ml microcentrifuge tube with 25 µl reaction volume containing 2 µl of DNA template (20 ng/µl), 0.5 µl of Taq DNA polymerase 0.2 mM of each dNTP, 2.0 mM $MgCl_2$ and

1 µl of decanucleotide primer (5 pmol/µl, Operon Technologies, Alameda, CA). The amplifications will be carried out by using a thermal cycler (MJ-Mini, Bio-Rad, USA) programmed at 94°C for 4 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, a final extension step at 72°C for 5 min and stored at 4°C. The amplified products will be resolved and visualized on a 1.4% agarose gel and photographed with Gel Doc XR system (Bio-Rad, USA). Variations in the banding patterns with missing or additional bands, intensity variation would be considered as criteria for DNA damage. This technique is now widely used to see the effect of environmental pollutants on organisms for determining DNA damage.

iii. Neurotoxicity study

Neurotoxic effects would be evaluated by studying the effect on acetylcholine esterase activity in test organisms by using the standard protocol of Ellman *et al*. (1961)

For acetylcholinesterase assays, 2-5 mosquito larvae will be homogenized in an Eppendorf tube containing 250 ml of extraction solution (100 mM sodium phosphate buffer pH 7.0, 1% Triton X-100). Tubes will be centrifuged at 10,000 g for 1 min and the supernatants will be used for the assays. The Ellman's spectrophotometrical method for cholinesterase activity determination utilizes 5, 5-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) as a chromogen. AChE hydrolyses the acetylthiocholine to generate thiocholine and acetate. The thiocholine in turn reduces the Dithiobis-Nitrobenzoic Acid liberating nitrobenzoate, which absorbs at 405 nm.

Risk assessment studies:

Considering the bio safety issues regarding the use of nanoformulations, risk assessment studies would be carried out. For this various nanopreparations would be tested on selected non target aquatic organisms such as *Daphnia*, snails etc.

Statistical analysis

Statistical analyses of the values for all experiments would be expressed as mean ± standard deviation. The data will be analyzed using Student's t test where statistical significance would be calculated using untreated (control) and nanoparticle treated samples and those with P value <0.05 would be considered as significant.

PLAN OF WORK

I PHASE

i. Fabrication of nanoformulations.
   - Chemical synthesis of AgNPs
   - Biological synthesis of AgNPs
   - Preparation of nanoemulsions

ii. Characterization of nanopreparations through TEM, SEM, XRD, FTIR, AFM, Zeta potential analyzer etc.

II PHASE

iii. Evaluation of efficacy against selected mosquito vectors following WHO (1996) protocols.

III PHASE

iv. Evaluation of cytotoxicity, genotoxicity and neurotoxicity in selected mosquito model.

IV PHASE

v. Evaluation of risk assessment in selected non- target organism.

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